

ADDITION OF ALPHA-LIPOIC ACID IN SEMEN EXTENDER IMPROVES POST-THAW ANTIOXIDANT PROFILE AND SEMEN QUALITY OF ACHAI (*BOS INDICUS*) CATTLE BULLS

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ABSTRACT

The effect of Alpha-lipoic acid (ALA) on lipid peroxidation, catalase activity and post-thaw semen quality of Achai bulls (*Bos indicus*) was studied. Semen samples (n=28), collected from adult Achai bulls (n=4) at weekly intervals, were diluted with Tris-citric acid egg yolk extender having various concentrations of ALA (0.00, 0.25, 0.50, 1.00 and 2.00mM) and frozen in liquid nitrogen (-196°C). Post-thaw semen assessment revealed that the lipid peroxidation decreased, while catalase activity increased (P<0.05) in all extenders containing ALA as compared to control, except in extender containing 2.00mM in which catalase activity decreased (P > 0.05) than control. Moreover, post-thaw semen quality parameters i.e., sperm viability and plasma membrane integrity were higher (P<0.05) in extender having 0.25 and 0.50 mM ALA compared to control, whereas mitochondrial membrane potential was significantly improved only with 0.50mM ALA as compared to control and all other ALA concentrations used. However, acrosome and DNA integrity of spermatozoa increased in extender supplemented with 0.25, 0.50 and 1.00mM ALA (P<0.05) compared to control. Computer Assisted Semen Analysis (CASA) of post-thaw semen revealed that total motility (%) and curvilinear velocity (µm/s), progressive motility (%), progressive fast motility (%) and beat cross frequency (Hz), and straight-line velocity (µm/s) and average path velocity (µm/s) were higher (P < 0.05) for 0.50 and 1.00mM, 0.25 and 0.50mM and 0.50 and 2.00mM ALA concentrations, respectively, than control. In conclusion, the addition of 0.50mM ALA in Tris-citric acid, fructose, egg yolk glycerol extender can perform optimum among all ALA concentrations (0.00, 0.25, 1.00 and 2.00mM) used, in terms of minimizing the oxidative stress and improving the post-thaw semen quality of Achai bulls.

Keywords: *Bos indicus*, Achai bulls, Alpha-lipoic acid, Post-thaw semen quality, Antioxidant enzyme, Computer Assisted Semen Analysis.

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INTRODUCTION

Achai is a dwarf-sized, dairy as well as light draught cattle breed, well-known for its better tolerability for harsh environmental conditions (Saleem *et al.*, 2013). The home tract of this breed is the Hindu Kush mountains of northern Pakistan extending to neighboring areas of Afghanistan. This breed can survive in the mountainous region under hilly and sub-hilly subsistence production system (Khan *et al.*, 2008). Milk produced by ~49.07% population of the Achai breed ranges between ~1501 to 2100 liters per lactation, while the remaining population (41.76%) produces milk around ~1001 to 1500 liters per lactation (Uddin *et al.*, 2014). Hence, there is a high scope of breed improvement in Achai cattle through artificial insemination (AI).

In Pakistan, conception rates through AI range from 38 to 62% (Anzar *et al.*, 2003). The problems related to AI services include sub-optimal semen processing, lack of farmers' awareness and skilled AI technicians, improper heat detection, and availability of liquid nitrogen for storage of frozen semen under field

conditions (Anzar *et al.*, 2003). During sperm cryopreservation, reactive oxygen species (ROS) like hydrogen peroxide, superoxide anion and hydroxyl radical are produced and deteriorate the sperm membrane stability. The unstable sperm plasma membrane results in altered motility patterns, reduced mitochondrial potential and abridged acrosome/ DNA integrity (Waterhouse *et al.*, 2010).

During the freeze-thaw process, about 50% of cells are damaged due to osmolality and oxidative stress (Watson, 2000). Oxidative stress to the spermatozoa is the major sequel of cryopreservation, results in irreversible damage to sperm organelles and changes in enzymatic profile, along with a reduction in sperm motility, plasma membrane integrity, and ultimately fertility (Bucak *et al.*, 2009a; Bucak *et al.*, 2009b). Polyunsaturated fatty acids are found in large quantities in sperm plasma membrane which makes it more vulnerable to oxidative stress (Alvarez and Storey, 1995). Nature has equipped the spermatozoa with an intracellular enzymatic defense mechanism to counter the ROS (Alvarez *et al.*, 1987; Lewis *et al.*, 1995). To cope

with this problem, the intracellular antioxidant system could be strengthened through addition of extracellular non-enzymatic antioxidants (Nair *et al.*, 2006). Several non-enzymatic antioxidants such as Taurine, Glutathione, Alpha-Lipoic acid (ALA), Trehalose, Methionine, Carnitine, and vitamin E and C have been used in bovine semen extenders to improve post-thaw sperm quality (Bucak *et al.*, 2010; Iqbal *et al.*, 2016).

Alpha-Lipoic acid is a short-chain fatty acid which is water and fat-soluble and is considered as a universal antioxidant due to its better permeability across the cell membrane (Ibrahim *et al.*, 2008). As high levels of ROS produced during cryopreservation damage cellular respiration (Bilodeau *et al.*, 2002), ALA helps in mitochondrial respiration (Lovell *et al.*, 2003) by enhancing Krebs cycle production of ATPs through pyruvate dehydrogenase and alpha-ketoglutarate dehydrogenase co-enzymes activation (Long *et al.*, 2009). Further, ALA protects against ROS by forming the metal layer around cell membrane (Packer *et al.*, 1995). It also enhances other antioxidants like vitamin C in the presence of glutathione (Ibrahim *et al.*, 2008). ALA has also been reported to increase sperm motility during in-vitro incubation of the bull spermatozoa (Ibrahim *et al.*, 2011). More specifically, it has been observed that ALA improves rapid forward progression and straight linear velocity, and limits ROS production (Ma *et al.*, 2011). Based on the observations, it was hypothesized that supplementation of ALA in semen diluent will improve the post-thaw sperm quality of Achai bulls. Therefore, the aims of the present study were: 1) to assess the oxidative stress through lipid peroxidation and catalase activity (as an indicator of antioxidant activity), and 2) to evaluate the post-thaw semen quality after addition of different concentrations of ALA in semen extender for Achai bulls.

MATERIALS AND METHODS

Animals and semen collection: Four adult Achai bulls, maintained at the Cattle Breeding and Dairy Semen Production Unit, Harichand, KPK (Latitude: 34°23'1.19"N and Longitude: 71° 48' 10.79" E) were used in the current study. All bulls were provided seasonal fodder at the rate of 10% body weight concentrates 2 to 3kg daily and water *ad libitum*. An artificial vagina (42°C) was used to collect semen from bulls (n=4) once in a week for eight weeks, with a total of 32 semen samples. The initial motility, volume and concentration of each ejaculate were assessed and the semen samples were pooled. After pooling, motility was again evaluated before further processing. One pooled sample having <65% motility was not further processed.

Semen Processing, Extension, and Freezing: A total of 100 ml Tris-citric acid, fructose, egg yolk glycerol

extender (pH 6.8; Tris; w/v 2.42%, citric acid 1.32% w/v, fructose 1% w/v; Merck, streptomycin sulphate 100 µg per ml, benzylpenicillin 1000 IU per ml; Sigma, glycerol 7% v/v and egg yolk 20% v/v) was prepared on each collection day and poured equally into five graduated tubes. Briefly, two molar stock solution of ALA was prepared by dissolving 2.06g of ALA in 5 ml distilled water and sodium hydroxide solution (Gohar *et al.*, 2014). Different concentrations i.e., 0.00 (control), 0.25, 0.50, 1.00, and 2.00mM of ALA were prepared by adding 0.00, 15, 30, 60 and 120 µl of stock solution into graduated tubes containing extender. Pooled semen samples were divided into five aliquots and added into graduated tubes kept at 37°C, so that final sperm concentration was 40 x 10⁶ live sperms per ml of extended semen in each tube. All samples were cooled to 4°C in a cold cabinet for 4 hours, filled in 0.5 ml French straws at the rate of 20 x 10⁶ live sperm per straw. All straws were initially exposed to liquid nitrogen (LN) vapors at -80°C for eight minutes and frozen into LN (-196°C). All straws were kept in LN for a minimum of one week and shifted to Theriogenology laboratory of University of Veterinary and Animal Sciences, Lahore for post-thaw semen analysis.

POST-THAW SEMEN EVALUATION

Catalase activity: Each frozen straw was thawed (37°C for 30 seconds) and spermatozoa were lysed using an ultrasonic liquid processor (ULP-750; Biotechnology Medical Services, USA), as described previously (Akalin *et al.*, 2016). After centrifugation (3000 rpm for 5 minutes), 50 µl supernatant was dispensed into a cuvette containing 500 µl of H₂O₂ (test sample), and 500 µl H₂O contained in another cuvette as a control test. Besides, cuvettes filled with 550 µl H₂O, and 500 µl H₂O₂ and 50 µl H₂O were kept as blank and standard, respectively. All the cuvettes (blank, standards, test, and control) were incubated at 37°C for 3 min and added with 2 ml of ammonium molybdate. Finally, 10 µl from each cuvette was examined for absorbance at 374nm, using a spectrophotometer (UV-2800; BMS; USA). Catalase activity in the sample was determined by using the following equation (Hadwan and Abed, 2016).

$$\text{Semen catalase activity:} \\ (KU) = \frac{2.303}{t} * \{\log S^{\circ}/S - M\} * VT/VS$$

Where t = total time, S^o = absorbance of standard tubes, S = absorbance of the test tube, M = absorbance of control test, VT = total volumes of reagents in test tubes, and VS = volume of semen

Lipid peroxidation: Malondialdehyde (MDA) concentration in frozen-thawed semen was measured with thiobarbituric acid assay (TBA), as described previously (Sohail *et al.*, 2018). Briefly, semen from each concentration of alpha-lipoic acid was thawed (37°C) and

mixed with 200 μl of 8% sodium dodecyl sulfate solution and 750 μl of 20% acetic acid in separate graduated tubes. Later, 1.5 ml of thiobarbituric acid and distilled water were added to make a total volume of 4 ml. The solution was then heated to 95°C for one minute and left to cool at room temperature for 30 minutes. Afterward, 5 ml butanol was added and the mixture was centrifuged at 4000 rpm for 15 minutes. Finally, a spectrophotometer (UV-2800; BMS; USA) was used to assess 100 μl of the supernatant layer for absorbance at 492 nm wavelength. The standard curve against known concentrations of Tetramethoxy Propane (TMP) i.e., 0.00, 0.78, 1.5, 3.1, 6.2, 12.5, 25, 50 and 100 $\mu\text{M/L}$ was utilized for calculation of MDA concentration ($\mu\text{M/L}$).

Sperm motility parameters, velocity distribution, and kinematics: A total of 10 μl of frozen-thawed semen from each concentration of ALA was evaluated for overall sperm motility, progressive motility and motility kinematics using a preheated glass slide and coverslip under 100 x magnification (AndroVision CASA system; Minitube, Germany). At least 400 spermatozoa per sample were counted in multiple microscopic fields. Sperm motility kinematics such as straight-line velocity (VSL $\mu\text{m/s}$), curvilinear velocity (VCL $\mu\text{m/s}$), average path velocity (VAP $\mu\text{m/s}$), linear distance (DSL μm), distance average path (DAP μm), amplitude of lateral head displacement (ALH μm), beat cross frequency (BCF Hz), linearity (VAP/VCL) and straightness (VSL/VCL) were analyzed. The computer-assisted semen analysis was performed with standard settings as followed: standard frame rate: 59; Spermatozoa were considered immotile when amplitude of lateral head displacement (ALH) < 1.0000 and curvilinear velocity (VCL) < 24.0000 $\mu\text{m/s}$. Spermatozoa were considered local motile when straight-line velocity (VSL) < 24.0000 $\mu\text{m/s}$ and VCL < 48.0000 $\mu\text{m/s}$. Spermatozoa were considered circular motile when path radius > 10.0000. Progressive motile spermatozoa had path radius > 10.0000 and VCL < 120.0000 $\mu\text{m/s}$.

Plasma membrane integrity (PMI%): Hypo-osmotic swelling test (HOST) was performed for the evaluation of PMI of spermatozoa. A 500 μl of HOS solution (190 mOsmol kg^{-1}) was mixed with 50 μl of semen from each concentration of ALA and incubated at 37°C for 30 minutes in a water bath. A total of one hundred spermatozoa were observed under phase-contrast microscope (400x; Olympus, Japan). Spermatozoa having an intact plasma membrane appeared swollen or coiled tailed (Khan and Ijaz, 2008).

Sperm Viability (%): Viability of spermatozoa was judged using Propidium Iodide (PI) under a fluorescent microscope (480/550 nm excitation/barrier filter; CX41 Olympus, Japan). A 50 μl Tris citric acid-fructose (TCF)

was mixed with 50 μl of semen from each concentration of ALA and centrifuged (3000 rpm for 5 minutes) to obtain a sperm pellet. A 2.5 μl of PI was added to the sperm pellet after resuspending it in TCF to a final volume of 47.5 μl and then incubated at 37 °C for 5 min. At least 200 spermatozoa were counted under multiple microscopic fields for viability. Non-viable sperm appeared red under the fluorescence light, however viable spermatozoa did not retain PI (Aquila *et al.*, 2011).

Acrosome integrity (%): Acrosome integrity was assessed by using normal apical ridge (NAR) solution consisting of 1% formal citrate (formaldehyde 37%; v/v and 2.9%, w/v trisodium citrate dehydrate; Merck). 50 μl of 1% formal citrate and 500 μl post-thawed semen were mixed and incubated for 30 minutes. At least 200 spermatozoa were observed under a phase-contrast microscope (400x; BX 51, Olympus, Japan). Spermatozoa with intact acrosome had a sharp black crescent on apical ridges (Rasul *et al.*, 2001).

Mitochondrial membrane potential: An equal volume (50 μl) of post-thawed semen and Tris citric acid-fructose (TCF) was mixed and centrifuged at 3000 rpm for 5 minutes. The sperm pellet was supplemented with TCF to make a total volume of 245 μl including 5 μl Rhodamine (RH-123, Sigma Aldrich, USA), and incubated (25°C) for 20 minutes. A 5 μl drop of semen was observed using a fluorescence microscope (480/550 nm excitation/barrier filter; CX41 Olympus, Japan). At least 200 spermatozoa were examined. Spermatozoa with normal intact mitochondrial membrane appeared green under fluorescence at the midpiece region (Dodaran *et al.*, 2015).

DNA integrity (%): DNA integrity was assessed using Acridine Orange (AO). Briefly, post-thawed semen (100 μl) and TCF (200 μl) were mixed, and centrifuged (3000 rpm for 15 minutes) to obtain a sperm pellet which was resuspended in TCF to make a smear on a glass slide. Air-dried semen smears were kept for 2 hours in Carnoy's solution followed by a Tampon solution for 5 minutes at 60°C. Finally, Semen smears were kept in an acridine orange solution for 5 minutes. A total of 200 spermatozoa were observed for the presence of green (intact DNA) or red (damaged DNA) color under a fluorescence microscope (CX41; Olympus, Japan; Tejada *et al.*, 1984).

Statistical Analysis: Values are expressed as mean \pm standard error of the mean. One way analysis of variances (ANOVA) was applied to analyze the effect of ALA on each semen quality parameter and differences between concentrations were analyzed by Duncan's multiple range test through SPSS (version 16.0) software.

RESULTS

The post-thaw profile of catalase (Ku/L) was higher ($P < 0.05$) at 0.50mM of ALA concentration as compared to control and all other concentrations. The difference among 1.00, 2.00mM ALA concentrations and control was non-significant ($P > 0.05$) in terms of catalase profile with minimum value for 2.00mM concentration. On the other hand, lipid peroxidation ($\mu\text{M/L}$) in all concentrations of ALA was lower ($P < 0.05$) than control with non-significant ($P > 0.05$) difference among all ALA concentrations (Table 1).

Post-thaw sperm viability percentage was improved ($P < 0.05$) by 0.25 and 0.50mM ALA concentrations as compared to control and other ALA concentrations, where 2.00mM significantly ($P < 0.05$) decreased sperm viability as compared to control and all ALA concentrations. Similar trend was observed for plasma membrane integrity percentage with exception of non-significant difference ($P > 0.05$) among 0.25 and 2.00mM ALA concentrations, and 2.00mM ALA with control. The difference among 0.25, 1.00, 2.00mM ALA concentrations and control in terms of mitochondrial membrane potential was found non-significant ($P > 0.05$) with maximum value observed for 0.50mM ALA concentration ($P < 0.05$). Moreover, acrosome integrity

percentage was higher ($P < 0.05$) at 0.25, 0.50 and 1.00mM ALA concentrations as compared to control, exhibiting non-significant difference ($P > 0.05$) among 0.25, 1.00 and 2.00mM ALA concentrations. In addition, DNA integrity percentage was significantly higher ($P < 0.05$) at 0.25mM, 0.50mM and 1.00mM ALA concentrations as compared to 2.00mM and control (Table 2).

Post-thaw total sperm motility, progressive motility and progressive fast motility percentages were improved ($P < 0.05$) with 0.50mM ALA as compared to 0.25mM, 1.00mM, 2.00mM concentrations and control, showing non-significant difference ($P > 0.05$) among 0.25, 1.00 and 2.00mM ALA concentrations. Additionally, the values for curvilinear velocity ($\mu\text{m/s}$) and average path velocity ($\mu\text{m/s}$) were higher ($P < 0.05$) at 0.50mM ALA as compared to control and other ALA concentrations. Moreover, the straight line velocity ($\mu\text{m/s}$) was higher for 0.50 and 2.00mM ALA concentrations as compared to control, with non-significant differences ($P > 0.05$) among 0.25, 1.00mM and control, and among all ALA concentrations. Beat cross frequency (Hz) was higher ($P < 0.05$) for 0.25 and 0.50mM ALA concentrations as compared to control, exhibiting non-significant difference ($P > 0.05$) among 1.00, 2.00mM ALA concentrations and control (Table 3).

Table 1. Effect of alpha-lipoic acid in semen extender on post-thaw semen enzymatic profile of *Bos indicus* (Achai) bulls.

Post thaw enzymatic profile	Concentration of Alpha-lipoic acid (mM)				
	0.00	0.25	0.50	1.00	2.00
Catalase activity (Ku/L)	23.61±4.00 ^c	34.82±4.95 ^b	49.21±2.77 ^a	24.80±3.77 ^{bc}	22.23±1.54 ^c
Lipid peroxidation ($\mu\text{M/L}$)	10.33±0.06 ^a	9.03 ±0.17 ^b	8.60 ±0.27 ^b	8.80 ±0.40 ^b	8.91±0.16 ^b

Different superscripts in a row indicate significant differences ($p < 0.05$) among groups.

Table-2. Effect of alpha-lipoic acid in semen extender on post-thaw semen parameters (motility, viability, plasma membrane, mitochondrial membrane potential, Acrosome, and DNA integrity percentage) of *Bos indicus* (Achai) bulls' spermatozoa. (n = 4 bulls and n = 28 ejaculates)

Semen quality parameters	Concentration of Alpha-lipoic acid (mM)				
	0.00	0.25	0.50	1.00	2.00
Sperm Viability (%)	44.21±1.14 ^c	47.57±0.86 ^b	51.07±0.87 ^a	44.92±0.46 ^c	41.64±0.38 ^d
PMI (%)	43.28±0.44 ^c	45.07±0.61 ^b	53.14±0.41 ^a	43.35±0.58 ^c	43.92±0.49 ^{bc}
Mitochondrial Membrane potential (%)	44.92±0.29 ^b	46.42±0.94 ^b	50.71±0.44 ^a	44.71±0.76 ^b	45.57±0.61 ^b
Acrosome Integrity (%)	48.42±0.44 ^c	50.14±0.57 ^{ab}	50.71±0.21 ^a	50.07±0.55 ^{ab}	48.71±0.58 ^{bc}
DNA integrity (%)	96.07±0.74 ^b	98.14±0.43 ^a	98.92±0.20 ^a	98.00±0.83 ^a	95.64±0.76 ^b

Values with different superscripts in row indicate significant ($P < 0.05$) difference among groups.

Table 3. Effect of alpha-lipoic acid in semen extender on post thaw CASA parameters (motilities, velocity distribution and kinematics) of *Bos indicus* (Achai) bulls' spermatozoa. (n = 4 bulls and n = 28 ejaculates)

Semen quality parameters	Concentration of Alpha-lipoic acid (mM)				
	0.00	0.25	0.50	1.00	2.00
Total motility (%)	41.53±0.68 ^c	45.80±0.80 ^{bc}	56.76±2.59 ^a	46.98±1.75 ^b	42.36±1.09 ^{bc}
Progressive motility (%)	33.47±1.78 ^c	39.32±1.08 ^b	49.40±2.12 ^a	38.29±1.44 ^{bc}	34.79±1.36 ^{bc}
Progressive fast motility (%)	13.67±1.60 ^c	20.40±1.71 ^b	27.79±1.75 ^a	16.66±1.22 ^{bc}	17.34±1.62 ^{bc}
Progressive slow motility (%)	19.59±0.76	18.33±1.84	21.54±1.80	20.72±1.24	16.50±1.63
Curvilinear velocity (µm/s)	54.47±2.97 ^c	63.62±2.01 ^{bc}	82.26±3.27 ^a	64.82±2.83 ^b	63.98±4.25 ^{bc}
Straight line velocity (µm/s)	22.01±1.51 ^b	26.03±1.66 ^{ab}	30.42±0.84 ^a	25.94±1.98 ^{ab}	27.10±1.61 ^a
Average path velocity (µm/s)	27.58±1.47 ^c	32.12±1.51 ^{bc}	40.03±1.02 ^a	32.19±2.00 ^{bc}	33.05±1.87 ^b
Linear distance (µm)	7.42±0.76	9.49±0.82	8.03±0.85	8.06±0.38	7.37±0.53
Distance average path (µm)	9.92±0.75	12.20±0.81	11.79±0.77	10.39±0.55	9.93±0.52
Amplitude of lateral head displacement (µm)	0.97±0.02 ^a	0.77±0.03 ^b	0.72±0.03 ^b	0.78±0.02 ^b	0.72±0.02 ^b
Beat cross frequency (Hz)	6.61±0.35 ^c	8.17±0.47 ^{ab}	8.79±0.76 ^a	7.49±0.29 ^{abc}	6.94±0.29 ^{bc}
Linearity (VSL/VCL)	0.40±0.01	0.40±0.01	0.37±0.01	0.40±0.02	0.42±0.01
Straightness (VSL/VAP)	0.79±0.01	0.80±0.01	0.76±0.01	0.80±0.01	0.81±0.00

Values with different superscripts in the row indicate significant ($P < 0.05$) differences among groups

DISCUSSION

The current study was designed to explore the effect of ALA on cryopreservation of Achai bull spermatozoa (*Bos indicus*). ALA is considered as universal antioxidant based on its scavenger effect for free radicals (Packer *et al.*, 2001). Further, catalase is considered as a major antioxidant enzyme that promotes the defense system of spermatozoa against oxidative stress. In post-thaw semen enzymatic evaluation, catalase activity improved significantly ($P < 0.05$). The optimal concentration of ALA was 0.50mM. The property of ALA to improve catalase activity seems to be due to enhanced glucose uptake by cells that results in further increment in nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate levels as reported previously in different organs of old aged rats (Arivazhagan *et al.*, 2000).

The level of oxidative stress can be evaluated by MDA, an end product of lipid peroxidation (LPO). The lipid peroxidation profile in all ALA treated groups was lower than control ($P \leq 0.05$). These findings are in accordance with the past study on rat sperm, in which alpha-lipoic acid supplementation of extender reduced oxidative stress and improved semen quality (Selvakumar *et al.*, 2006).

During post-thaw semen evaluation, sperm motility is considered as an important parameter. It is believed that the sperm cells with rapid forward movement have greater capability to reach the site of fertilization. ALA acts as an antioxidant, as well as coenzymes of ATPs based on various enzyme systems like alpha-ketoglutarate dehydrogenase and pyruvate dehydrogenase. This results in increased energy availability to spermatozoa by enhanced mitochondrial

membrane potential and ultimately leads to increased sperm motility (Bilska and Wlodek, 2005). The results of this study revealed that pre-freeze treatment of semen extender with 0.50mM and 0.25mM ALA significantly increased post-thaw semen quality in terms of sperm motility, viability, and plasma membrane integrity ($P \leq 0.05$). These results are in accordance with previous reports which indicate improved semen quality following the addition of ALA in extender for cattle (Ibrahim *et al.*, 2011), buffalo (Gohar *et al.*, 2014), and buck semen (Ibrahim *et al.*, 2008). The ability of ALA to improve semen quality has also been demonstrated by feed supplementation in rats (Selvakumar *et al.*, 2006; Othman *et al.*, 2012; Yeni *et al.*, 2012). However, the results of current study are not in line with an earlier study on equine semen (Hussain *et al.*, 2011), where ALA showed no beneficial impact on post thaw semen quality parameters.

For active motile sperms, healthier mitochondria with high membrane potential are obligatory (Suarez *et al.*, 2007; Ahmed *et al.*, 2016). It has been reported that ALA promotes mitochondrial respiration (Lovell *et al.*, 2003), and results in additional ATPs production (Long *et al.*, 2009) that results in improved DNA integrity. These observations are in agreement with our data, which shows that the addition of ALA in semen extender results in improved mitochondrial membrane potential and DNA integrity.

In the current study, post-thawed computer-assisted semen analysis of semen diluted with extender containing 0.50mM ALA concentration showed significant improvement ($P \leq 0.05$) in quality parameters like total motility, progressive motility, velocity distribution, and kinematics. A previous study conducted on bovine (Bucak *et al.*, 2015) semen reported

significantly enhanced post-thawed sperm motility in an extender containing 1.0mM ALA in combination with 2.0mM cysteamine. Moreover, increasing the concentration of ALA decreased sperm motility which was attributed to the change in pH of the sperm environment towards high acidic, which is detrimental to sperm and results in immobility (Olmsted *et al.*, 2000). In our study, the best post-thaw semen quality was obtained in semen extender containing 0.50mM ALA, which is a lower concentration in comparison with 1.0mM used in an earlier study (Bucak *et al.*, 2015).

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